

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Eric A. Schon

Serial No.: 08/409,644

Examiner: J. Fredman

Filed: March 24, 1995

Group Art Unit: 1807

For: A METHOD TO DETECT MUTATIONS IN A NUCLEIC ACID
USING A HYBRIDIZATION-LIGATION PROCEDURE

1185 Avenue of the Americas
New York, New York 10036
February 29, 1996

Assistant Commissioner of Patents
Washington, D.C. 20231

DECLARATION OF ERIC A. SCHON PH.D. UNDER 37 C.F.R. § 1.131

Sir:

I, Eric A. Schon, hereby declare as follows:

1. I am the sole inventor named on the above-identified patent application.
2. The invention claimed in the above-identified application was conceived solely by me, and either directly or through persons acting under my direction and supervision, actually reduced to practice in the United States prior to September 30, 1994.
3. As evidence of the fact that the invention claimed was actually reduced to practice in the United States prior to September 30, 1994, I have annexed hereto as Exhibits 1-4 copies of pages from the laboratory notebook of my technician, Mr. Jeffery S. Rogers. These copies are true and accurate copies except that the dates have been

Eric A. Schon
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Exhibit C

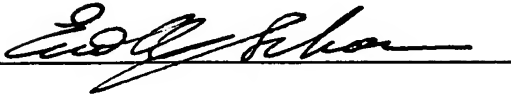
redacted. All of the redacted dates are prior to September 30, 1994.

4. The claimed invention is a method for detecting the presence or absence of a predefined mutation in a nucleic acid. This method was specifically used to detect the presence of a known mutation in the MELAS-3243 nucleic acid. The claimed invention involves contacting the nucleic acid molecule with a linear probe comprising two covalently linked nucleic acid segments under conditions such that the unlinked end of each segment of the probe is capable of hybridizing with the nucleic acid molecule. As shown in Exhibit 1, a probe designated the LiCat-Melas.1 probe was synthesized and radiolabeled. As shown in Exhibit 2, the mutant MELAS-3243 nucleic acid was contacted with the LiCat-Melas.1 probe, under suitable hybridization conditions to form a hybridization product.
5. The claimed invention further involves contacting the hybridized product from paragraph 4 above, with a ligase under conditions such that the unlinked ends of the segments ligate together if the nucleic acid molecule contains the mutation. As shown in Exhibit 3, the hybridization product was contacted with T4 DNA ligase under suitable ligation conditions.
6. The claimed invention further involves determining whether the unlinked ends of the segments have ligated together so as to thereby detect the presence or absence of the mutation in the nucleic acid molecule. As shown in Exhibit 4, the hybridization product was then electrophoresed through a 0.8% agarose gel, dried under vacuum and then subjected to autoradiography in order to

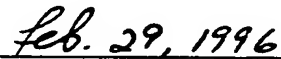
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determine whether ligation has occurred. Ligation had occurred. In this way the presence of the predefined mutation in the nucleic acid molecule was detected.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of United States Code and that false statements may jeopardize the validity of the application or any patent issuing thereon.



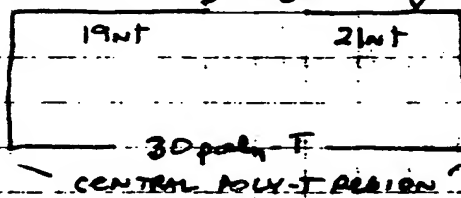
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Date

Biotinylate dTTP
control nt's?

5' TAIL 5' 3' AA ← A² removed ← 3' TAIL



Li CAT-MELAS.

5' end TAIL

CENTRAL 'POLY-T' REGION

$5' - \text{CTG} / \text{CCA} / \text{TCT} / \text{TAA} / \text{CAA} / \text{ACC} / \text{CTT} / \text{TTT} / \text{TTT} / \text{TTT} / \text{TTT} / \text{TTT} /$
 $\text{TTT} / \text{TTT} / \text{TTT} / \text{GGT} / \text{TTT} / \text{ATG} / \text{CGA} / \text{TTA} / \text{CCG} / \text{GGC} / \text{C} - 3'$

These 2 T³ replace A³ which were originally part of 3¹ T³ L.

* ordered from DNA Synthesis Facility

→ There may be a problem with synthesizing a primer with this many polynucleotides (TTP). Sometimes chain will fold back on itself and fail to extend during synthesis. → assays can do this with no problem.

TEMPLATES

PCR 14.3/16.4 would be good. (planned is 5.3kb) - control.

maio 2013 +
Patient DMT's: Stow | Fenofibrate | Nardi | Ramipril Available?

-obtained from Yassin: WS176: ~~meas~~ + Cyband (nonoplastic)
WS239: ~~meas~~ + Cyband (nonoplastic)

II. CLEAN LABELED PRIMER: (Maniatis technique Book III pg. E37-E38)

1. Add 80 λ TE to reaction tube (final volume = 100 λ)
2. Spin out TE of prepared G-25 column: (G-25 Sephadex - Medium)
 - a. 1ml tuberculin syringe \rightarrow plug bottom w/ glass wool
 - b. Add G-25 slurry to syringe (make sure G-25 doesn't flow out)
 - c. Spin syringe on table top centrifuge (Speed #3) 2' (Place syringe in 15ml Corning tube for support)
 - d. Repeat until packed column (looks slightly deaerated is 4.15" high)
 - e. Add fresh TE to top of column + spin through. Repeat this step 3-4 times to wash column.
 - f. Plug top with white cap. Add fresh TE to top of column. Parafilm top. Store +40°C in upright stance.

3. Add 100 λ of Primer R+V mix to top + center of stacked G-25 bed.
4. Place syringe in fresh microfuge tube.



5. Spin column on #3 x 4'
6. Check tube for collected 100 λ \rightarrow this is labeled product
7. Check column for collected radioactivity + check product.

MEASURE SPECIFIC ACTIVITY:

1. Estimate volume. Add 1 λ of product to 5 μ l Scintillation Solu
2. Measure on Spec: = 189745 CPM / λ x 100 λ = 1.9×10^7 CPM

P#	S#	TIME	CPMA/K	%DEV	CPMB/K	%DEV	CPMC/K	%DEV	SIE	SIS F
6	1	1.00	189745.	.46	158625.	.50	.00	.00	.000	690.02
6	Blank 2	1.00	20.00	44.7	7.00	75.5	.00	.00	.000	19.716
			94882.5		79315.0		.00		.000	354.86
6	¹⁴ C 3 STD	1.00	112084.	.60	54382.0	.86	.00	.00	.000	171.15
6	STD. 4	1.00	27.00	38.4	16.00	50.0	.00	.00	.000	25.563
			56055.5		27199.0		.00		.000	98.357

$$\frac{1.9 \times 10^7 \text{ CPM}}{9.2 \text{ ug}} = 2 \times 10^6 \frac{\text{CPM}}{\text{ug}}$$

(40 pmol)

$$2 \times 10^6 \frac{\text{CPM}}{\text{ug}}$$

$$4.8 \times 10^5 \frac{\text{CPM}}{\text{pmol}}$$

40 pmol
100 λ

0.4 pmol/ λ (10 pmol = 25 λ)

LABELED PRIMER CONC

HYBRIDIZATION

(10:1) Primer / Template Ratio PCR 16.3/16.4 ONLY

IN tube: 25 λ LICAT-MELTS.1 Primer (10 pmol)
1 λ PCR 16.3/16.4 ($\approx 1.5 \mu\text{g} = \approx 1 \text{ pmol}$)
10 λ 10X ANNEALING Buffer (See Below)
64 λ DDW

100 λ TOTAL

Boil x 5' (Denature Plasmid DNA)



55°

2 HRS

Eric thinks primer/template annealing will be rapid. \rightarrow suggested shorter time to prevent plasmid re-annealing.

1 pmol = 7 μg

LICAT-MELTS 1 pmol = 0.23 μg

PCR 16.3/16.4 1 pmol = 1.8 μg

10X ANNEALING BUFFER

500 mM NaCl

100 mM MgCl_2

100 mM TRIS-CL pH 7.4

1. Remove hybridization tubes from 55°C bath \rightarrow Place on ice.
2. Add 0.2 x vol of 10M NH_4OAc (20 λ) \rightarrow mix
3. Add 240 λ ice-cold 100% Ethanol \rightarrow mix. \rightarrow Store on ice 15-30' (The hybridized DNAs will co-precipitate as a unit. Along with un-hybridized DNAs.)
4. Centrifuge 15' @ 13,000 RPM.
5. Remove S°. Wash gently 2x w/ RT 70% Ethanol (check pellet for radioactivity)
6. Resuspend pellet in 10 λ DDW.

remove 5x of this mixture and store -20°C for step control

Layers 1 A

cont.

oops → mistake mode 8/10/1

(1:1 PRIMER (TEMP RATIO))

To lyophilized pellet add:

2λ LICAT-MEAS. 1 Primer (0.8 pmol) (1/29 Sample) ^{check}

2λ 10X Hyb. Buffer

16λ DDW

65°C x 2' → Slow Cool < 35°C x 45'

↓

↓

same 5λ

Spin briefly

(+Hyb
-Lig.)

LIGATIONS

+CONTROL

5λ 2HINDIII DNA (0.5 μg)

1λ T4 DNA Ligase

2λ 10X Ligase Buffer

12λ DDW

20λ

10λ Template / Primer Ancestral mix

2λ 10X Ligation Buffer (5m)

1λ T4 DNA Ligase

7λ DDW

20λ

12-15°C Bath x ~~24 hrs~~ O.N. (2:20 PM → 10:30 AM)

~20 HRS.

→ Inactivate Ligase → 65°C x 15'

Remove 10λ of ligation mix to separate tubes (+Lig
+Hyb)

Of the remaining 10λ:

Add

10λ mix

0.8λ 5mM EDTA

4λ 1N NaOH

5.2λ DDW

RT x 15' on ice

+2λ 10X Dye

Load denatured DNA directly into gel
do not delay.

PREPARE 0.8% TAE AGAROSE GEL:

LANE/LOAD

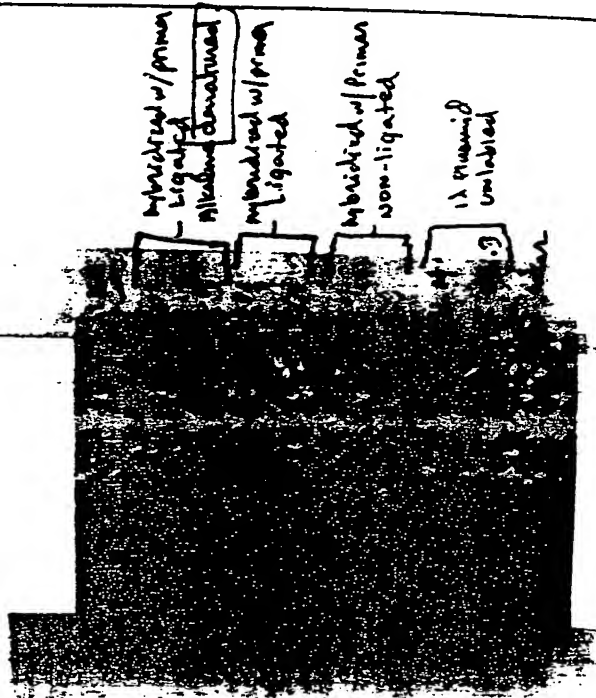
- 1 - λ HIND III Marker (0.5 μ g of λ HIND III used in ligation + cont.)
- 2 - PCR 16.3 12 Plasmid
- 3 - PCR 16.4 "
- 4 - PCR 16.3 + HYB / -LIG (No Denature) (Use half)
- 5 - PCR 16.4 + HYB / -LIG (No Denature) (Use half)
- 6 - PCR 16.3 + HYB / +LIG (")
- 7 - PCR 16.4 + HYB / +LIG (")
- 8 - PCR 16.3 " " + Alkaline Denatured
- 9 - PCR 16.4 " " " "
- 10 - LIGATION + CONTROL

~~2ND ROW~~

- ~~1 - λ HIND III Marker~~
- ~~2 - PCR 16.3~~
- ~~3 - PCR 16.4~~
- ~~4 - PCR 16.3 + HYB / -LIG Denatured (Alkaline)~~
- ~~5 - PCR 16.4 + HYB / -LIG " "~~

cell dried on vacuum 2+RS.

expose + screen
-80°C



WITNESSED: *[Signature]*

RESULTS

IT WORKS! the catenanes of pCR16.3 (meats+) + LICAT meats.
 Primer appears to be resistant to denaturation + loss of signal.
 Even the 650C Ligase killing step (post-ligation) makes the
 wt template lose the primer, but not the meats+ template

NEXT STEP:

- 1) Repeat experiment using hybrid total DNA's
 (Ligand RKN → hyb / lig. etc.) meats+
 meats-
- 2) Spot solid matrix (e.g. Zeta-probe or membrane)
 with primer + hyb / ligate / denature with previous
 labeled primer. (same matrix technique previous)
 ? 1 membrane material interfere with
 ligation reaction??
- ? 2 Do reactions on punched holes
 of membrane in 96-well TC dish!
- ? 3 STRIPS of membrane?

WS176 = meats+ } both
 WS239 = wt } homoplasmic

LICAT.MEATS.1 + CYBRID TOTAL DNA

I. SPECTROPHOTOMETER MEASURE (@37°C)

	IN TE PH8	A ₂₆₀	A ₂₈₀	
1. WS176 (MEATS+)	1:1000 Diln.	.044	0	440.D.
2. WS239 (WT)	1:1000 Diln.	.010	0	100.D.

meats+ : 2200 ug/me = 2.2 ug / λ TOTAL DNA

WT = 500 ug/me = 0.5 ug / λ " "

say, roughly 10% of yield
 is from mtDNA

0.20 ug / λ

0.05 ug / λ

mtDNA yield

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